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ÚLOHA TOLL-LIKE RECEPTORŮ V PATOGENEZI JATERNÍCH ONEMOCNĚNÍ

THE ROLE OF TOLL-LIKE RECEPTORS IN THE PATHOGENESIS OF LIVER DISEASES

Autoreferát disertační práce/Ph.D. thesis – short report

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1. Summary

A majority of chronic and acute liver diseases share a significant degree of liver inflammation and injury attributable to innate immunity, activated through Toll-like receptors (TLRs). W investigated the involvement of TLRs on the pathogenesis of alcoholic liver disease, non-alcoholic steatohepatitis and immune-mediated liver injury.

In a multicentric study in Central European region involving more than 370 patients with alcoholic liver cirrhosis (ALC) and 700 controls, we investigated genetic susceptibility to ALC. We asked whether the risk of ALC could be modified by allelic variants in key molecules involved in TLR4 signaling, which is induced by the gut-derived lipopolysaccharide (LPS). We did not find any association of any alleles under the study with ALC. A significant association of one of the investigated alleles was found only after our data were included in a meta-analysis. However, the relative risk attributable to this allelic variant was below any biological importance. Our data indicated that the contribution of the studied alleles to genetic susceptibility to ALC is low, and suggested existence of other TLR-dependent mechanisms that could mediate the damaging effect of alcohol/LPS in the liver.

Therefore, using a mouse model, we investigated the role of the interferon-regulatory factor 3 (IRF3) in the pathogenesis of alcohol-induced liver injury. IRF3, an alternative downstream mediator of TLR4 activation, induces inflammatory cytokines and Type I interferons (IFNs). We found that IRF3 deficient mice were protected from alcohol-induced liver injury. As IRF3 is expressed both in liver macrophages and hepatocytes, we generated chimeric mice with selective *Irf3* deficiency in bone-marrow derived cells or in liver parenchymal cells. We identified that the pro-inflammatory effect of IRF3 in alcoholic liver injury is specific to bone marrow-derived cells, supporting the crucial role of TLR4/IRF3-mediated inflammation in alcohol-induced liver injury. In contrast, we showed that IRF3 in parenchymal liver cells has a protective role. This effect is mediated by hepatocyte-derived Type I IFNs that induce Type I IFN-dependent anti-inflammatory cytokines in liver mononuclear cells which in turn modulate the extent of liver inflammation and injury.

Having demonstrated the differential effects of TLR4 signaling in the liver, we asked whether TLR4 downstream pathways could be amenable to therapeutic intervention in NASH, which is dependent on gut-derived LPS. We hypothesized that modification of gut microflora by a probiotic diet would ameliorate diet-induced NASH in mice. Administration of the VSL#3 probiotic diet failed to prevent liver steatosis or inflammation. In contrast, VSL#3 ameliorated liver fibrosis in NASH. Analysis of fibrogenic pathways revealed a modulation of transforming growth factor-β signaling and collagen expression in the liver by the VSL#3 diet. These results suggested that the benefit of the VSL#3 probiotic treatment on fibrosis may occur even in the absence of significant changes in markers of inflammation and fat in the liver.

In addition to TLR4 activation, a number of liver diseases, including autoimmune, exhibit induction of inflammatory TLR9 signaling, triggered by DNA from gut-derived bacteria or from dying host cells. In addition to inflammatory cytokines, activation of TLR9 induces a strong Type I IFN response. In a mouse model of TLR9-associated liver injury, we showed that genetic deficiency of Type I IFN induction or signaling exaggerated liver damage and inflammation, and increased production of TNF-α by liver mononuclear cells. Mice deficient in Type I IFNs showed decreased expression of the interleukin 1-receptor antagonist (IL-1ra), which is a Type I IFN-inducible anti-inflammatory cytokine. IL-1ra protected cultured hepatocytes from IL-1β-mediated sensitization to cytotoxicity from TNF-α. Moreover, administration of exogenous Type I IFN or IL-1ra ameliorated TLR9-associated liver injury, implying that the endogenous anti-inflammatory signaling induced by Type I IFNs and mediated by IL-1ra regulates the extent of TLR9-induced liver damage.

In conclusion, our data demonstrate a cell-specific role of IRF3 in the pathogenesis of alcohol-induced liver injury and support the importance of the TLR4-dependent/MyD88-independent signaling in alcoholic liver disease. Furthermore, our novel findings emphasize the active role of hepatocytes in modulating the extent of the innate immune response in the liver. Also, we demonstrate that the endogenous anti-inflammatory signaling induced by Type I IFNs regulates the extent of liver damage induced by the innate immunity, and support the indispensable role of Type I interferon signaling in TLR-mediated liver injury. Lastly, we suggest a potential role for IL-1ra in therapy of liver diseases with inflammatory component induced by TLR signaling.

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2. Introduction

Toll-like receptors (TLRs) are a highly conserved group of pattern recognition receptors that function as pathogen sensors. The recognition of specific pathogen-associated molecular patterns (PAMPs) by TLRs is a cornerstone of the innate immune system, and enables it to rapidly mount protective responses against invading pathogens.

The human TLR family consists of 10 members, which enable the innate immune system to discriminate between PAMPs and launch specific defense mechanisms. Specifically, TLR4 recognizes lipopolysaccharide (LPS, endotoxin) from Gram-negative bacteria, TLR2 is essential for recognition of microbial lipopeptides, such as lipoteichoic acid (LTA) and TLR9 recognizes CpG-rich DNA derived from bacteria or mammalian apoptotic cells. TLR1 and 6 with TLR2 distinguish between triacyl- and diacyl-lipopeptides, TLR3 recognizes viral double-stranded RNA, and TLR5 recognizes bacterial flagellin. TLR7 and TLR8 bind viral single stranded RNA.

Toll-like receptors play a major role in liver pathophysiology due to the liver's anatomical association with the intestine and its exposure to relatively large amounts of intestinally derived PAMPs. Both non-parenchymal and parenchymal cells in the liver express TLRs. Kupffer cells (resident liver macrophages) express TLR2, TLR4, TLR9 and TLR3, hepatic stellate cells (HSC) express TLR2, TLR4 and TLR9, and liver sinusoidal endothelial cells express TLR4. Hepatocytes express all TLRs (1).

Recognition of LPS occurs through TLR4, which is a membrane receptor expressed predominantly on Kupffer cells. Activation of TLR4 by LPS requires the presence of the co-receptor CD14 for signaling. TLR4 activates two distinct signaling pathways. One pathway is activated by the adaptor MyD88, which leads to activation of nuclear factor κB (NF-κB) and to the induction of pro-inflammatory cytokines, such as tumor necrosis factor α (TNF-α) and interleukin-1β (IL-1β). The second pathway (MyD88-independent) is mediated by the interferon regulatory factor 3 (IRF3) that induces inflammatory cytokines and Type I interferons (IFN-α and IFN-β) (2).

The adaptor MyD88 is critical also in mediating signals from TLR2 and TLR9. TLR2 is a membrane receptor recognizing LTA whereas TLR9 is endosomal receptor that binds CpG-DNA. Activation of TLR2 or TLR9 results in production of inflammatory cytokines via the NF-κB. In addition to inflammatory cytokines, binding of CpG-DNA to TLR9 activates the interferon regulatory factor 7 (IRF7), which is a strong inducer of Type I IFNs (IFN-α and IFN-β) (3).

2.1. Toll-like receptor 4 (TLR4)-mediated signaling in the pathogenesis of alcohol-induced liver disease

Alcoholic liver disease (ALD) is the most common drug abuse-induced liver disease and accounts for 40% of deaths from cirrhosis in the United States. Gut-derived LPS is a key player in the pathogenesis of ALD. Excessive alcohol intake changes the intestinal epithelial barrier causing increased intestinal permeability followed by elevated LPS levels in the portal circulation. The LPS then activates TLR4 on Kupffer cells to produce proinflammatory cytokines, leading to hepatocyte damage. Indeed, treatment with *Lactobacillus sp.* or antibiotics suppresses alcohol-induced liver injury by changing enteric microflora. Kupffer cell-inactivated animals, CD14- or TLR4-deficient mice and mice deficient for TNF-α or IL-

1β have a strong reduction of liver injury despite elevated endotoxin levels, supporting the crucial role of TLR4 signaling and TLR4-downstream effects in the pathogenesis of ALD (4).

2.1.1. The role of allelic variants in TLR4-mediated inflammatory pathways in genetic susceptibility to alcoholic liver cirrhosis

Only 40% of heavy drinkers will develop alcoholic steatohepatitis and only 5% will develop cirrhosis (ALC). In addition to alcohol, gender and comorbidities, yet unidentified genetic factors account for at least 50% of the individual susceptilibity to ALC (5).

A majority of genes coding for receptors and signaling molecules involved in TLR4 signaling shows a high frequency of functionally relevant allelic variants. For example, the -159C/T variation in the promoter of *CD14* enhances its expression by monocytes, whereas two linked variations, c.896A/G and c.1196C/T, in the coding region of *CD14* impede the activation of monocytes by LPS. The variation -238G/A in the *TNFA* promoter increases transcription of TNF-α. The *IL-1* gene cluster contains *IL1B* and *IL1RN* genes, which encode IL-1β and its receptor antagonist IL-1ra, respectively. The variant –31T in the promoter of *IL1B*, significantly increases IL-1 transcription. The IL1RN gene contains a penta-allelic 86-bp tandem repeat. The second most common IL1RN*2 allele containing two repeats increases the secretion rate of IL-1β *in vitro* (6).

Considering the importance of inflammatory activation in the pathogenesis of alcohol-induced liver injury, which is induced by LPS, we tested the hypothesis that allelic variants of genes involved in TLR4 signaling and TLR4-mediated downstream effects will be associated with the susceptibility to ALC. We tested this hypothesis by means of large case-control association study conducted on two Central European populations.

2.1.2. The role of Interferon regulatory factor 3 (IRF3) and Type I interferons in the pathogenesis of alcohol-induced liver injury

Recent evidence demonstrates that TLR4 downstream signaling in ALD is mediated predominantly through the MyD88-independent pathway, rather than through the MyD88-dependent mechanism. The involvement of the MyD88-independent, IRF3-dependent signaling was indicated by upregulation of IRF3-inducible genes in Kupffer cells isolated from mice fed with alcohol (2).

Based on the emerging role of MyD88-independent downstream pathways in TLR4 signaling and in ALD, we hypothesized that IRF3 is critical in alcohol-induced liver injury. Given the differential input of parenchymal and non-parenchymal cells in pathophysiology of ALD, we further hypothesized that IRF3 may be critical in alcoholic liver injury in a cell-specific manner. Therefore, we employed a mouse model in which the effect of chronic alcohol feeding on liver damage was evaluated in animals with global deficiency of IRF3 or in animals with selective deficiency of IRF3 in liver parenchymal and non-parenchymal cells.

2.2. The role of probiotics in TLR4-mediated pathogenesis of non-alcoholic steatohepatitis

Nonalcoholic fatty liver disease and its advanced stage, nonalcoholic steatohepatitis (NASH), are becoming the most common causes of chronic liver disease in Western countries. Recent reports demonstrate that high fat diet and obesity alters the composition of caecal microbiota in favor of LPS-containing strains, and increases the gut permeability,

resulting in increased bacterial translocation. The key role of LPS and TLR4 signaling in NASH is supported by reduced hepatic triglyceride content and inflammation in mice undergoing intestinal decontamination with antibiotics, or in mice deficient for TLR4 (7). These findings emphasize the crucial role of gut-derived bacterial products in the pathogenesis of NASH and suggest that modification of intestinal microbiota by probiotics may represent a feasible approach for the prevention and treatment of NASH.

To test the hypothesis that modification of intestinal microbiota would have impact on NASH, we employed a mouse model of NASH which were administered with the VSL#3, a probiotic preparation of live, freeze-dried bacteria containing eight bacterial species (*Streptococcus salivarius* subsp. *thermophilus*, *Bifidobacterium* [*B. breve, B. infantis, B. longum*], *Lactobacillus acidophilus*, *L. plantarum*, *L. casei*, and *L. delbrueckii* subsp. *bulgaricus*). We asked whether the VSL#3 probiotic preparation, which has been previously shown to prevent hepatic damage and maintain colonic barrier function in a mouse model of sepsis (8), would ameliorate diet-induced NASH by modulation of liver inflammation and fibrosis.

2.3. Type I interferons in the pathogenesis of TLR9-associated liver injury

Emerging data provide an evidence for the role of CpG DNA and TLR9-mediated inflammation in acute and chronic liver injury of diverse origin, including alcoholic liver disease (9), primary biliary cirrhosis (10), primary sclerosing cholangitis (11) and acetaminophen-induced liver injury (12). TLR9-initiated signals are also involved in liver fibrosis (13), ischemia-reperfusion injury (14), and liver graft rejection (15). TLR9 acts synergistically with the TLR2 ligand LTA (16), and sensitizes to liver injury induced by the TLR4 ligand LPS (17).

Activation of TLR9 by CpG results in increased production of inflammatory mediators via NFκB, and in a strong induction of Type I IFNs (IFN-α and IFN-β) via the transcription factor IRF-7. Whereas the induction of inflammatory cytokines by CpG is considered the key event in the TLR9-mediated liver injury, little is known about the role of IRF7-dependent Type-I IFN induction and signaling after TLR9 stimulation (18). Using mice deficient for IRF7 and Type I IFN receptor α/β (IFNAR1), we investigated the role of Type I IFNs and Type I IFN-mediated signaling in TLR9-induced liver inflammation and injury.

3. Specific aims

To gain insight into the downstream effects of Toll-like receptors in alcohol-induced liver injury, non-alcoholic steatohepatitis and Toll-like receptor 9 –associated liver injury, we proposed the following specific aims:

A. To elucidate the role of common allelic variants in genes involved in Toll-like receptor 4-mediated liver injury in genetic susceptibility to alcoholic liver cirrhosis.

B. To determine the cell-specific effect of the Interferon regulatory factor 3 (IRF3) in the pathogenesis of alcohol-induced liver injury

C. To investigate the role of probiotics in modulation of liver inflammation, injury and fibrosis in the pathogenesis of non-alcoholic steatohepatitis

D. To assess the role of Type I interferons in the pathogenesis of Toll-like receptor 9 –induced liver injury

4. Methods

4.1. General methods

Nucleic acid and protein isolation from human and animal cells and tissues
Restriction analysis of DNA
Reverse transcription of total RNA (generation of cDNA)
Polymerase chain reaction, quantitative real-time polymerase chain reaction
DNA electrophoresis on agarose and polyacrylamide gel
Enzyme-linked immunoabsorbent assay (ELISA)
SDS-PAGE protein electrophoresis and protein immunobloting (Western blotting)
Biochemical analysis of alanine aminotransferase and lactate dehydrogenase activity
Cell culture techniques
Flow cytometry
Immunohistochemistry
Experimental work with small laboratory animals, including in situ liver perfusion and cell isolation
Descriptive statistics, parametric and non-parametric tests of statistical hypotheses, power calculation, regression analysis
Statistical methods in genetics and in genetic epidemiology

4.2. Specific methods

4.2.1. Methods related to genetic susceptibility to alcoholic liver cirrhosis

DNA genotyping. Genomic DNA was isolated from peripheral blood using the QIAamp DNA Blood Mini Kit (Qiagen GmbH, Hilden, Germany). Single nucleotide allelic variants were

determined by polymerase chain reaction (PCR) followed by restriction fragment length (RFLP) analysis of the products. Genotyping of the *IL1RN* variable number of tandem repeats (VNTR) locus was performed by a PCR-based fragment length polymorphism method. Restriction endonucleases were purchased from Fermentas (Fermentas UAB, Vilnius, Lithuania). After testing for Hardy-Weinberg equilibrium (HWE), allele frequencies were checked for consistency with data from the population of European ancestry [Utah Residents with Northern and Western European Ancestry (CEU)] from the HapMap database (www.hapmap.org).

Statistical analysis. Two-sided power calculations at p=0.05 for 80% statistical power were performed using the DSTPLAN software (http:// linkage.rockefeller.edu/soft). When the odds ratio (OR) of a polymorphism was assumed to be 2, the required sample size was 100 cases and 180 controls for the polymorphism with frequency of 0.5 (*CD14* –159C/T). When the OR was assumed to be 4, the same sample size was sufficient to detect a true effect of a polymorphism with frequency of 0.03 (*TNFA* –238G/A). HWE of alleles at individual loci was evaluated using the program HWE (http://linkage.rockefeller.edu/soft). Haplotype frequencies for pairs of alleles and linkage disequilibrium (LD) coefficients $D'=D/_{Dmin\ or\ max}$ and r^2 were calculated using the Arlequin software (19). Age and median alcohol consumption between the groups was compared using the Mann-Whitney test. Male to female ratios were compared using the Fisher exact test. All association analyses were performed by logistic regression analysis using SPSS software version 14.0 (SPSS Inc., Chicago, IL, USA). Where applicable, logistic regression analysis adjusted for age was performed.

4.2.2. Methods related to the role of IRF-3 in the pathogenesis of alcohol-induced liver injury

Animal and experimental protocol. Six to eight-week-old, female C57Bl/6 wild-type, IRF3-deficient (IRF3-KO) and Type I interferon α/β receptor 1-deficient (IFNAR-KO) mice (kind gift of Jonathan Sprent, Scripps Research Institute, La Jolla, CA), were employed. Chimeric mice were generated by transplanting wild-type (C57Bl/6) bone marrow into irradiated WT mice (WT/WT-BM) o IRF3 deficient mice (IRF3-KO/WT-BM), or by transplanting IRF3 deficient bone marrow into WT mice (WT/IRF3KO-BM). Some animals were fed with the Lieber-DeCarli diet (Dyets, Inc., Bethlehem, PA) with 5% (vol/vol) ethanol (36% ethanol-derived calories) for 4 weeks; pair-fed control mice matched the alcohol-derived calories with dextran-maltose. Serum was stored at -80°C. Livers were snap-frozen in liquid nitrogen for proteins, or stored in RNAlater (Qiagen GmbH, Hilden, Germany) for RNA extraction, or fixed in 10% neutral-buffered formalin for histopathological analysis.

*Biochemical assays.*Serum alanine aminotransferase (ALT) was determined using a kinetic method (D-Tek LLC., Bensalem, PA). Liver triglyceride levels were assessed using the L-Type Triglyceride H kit (Wako Chemicals USA Inc., VA).

Cytokine measurement. Mouse IL-1β ELISA kit was purchased from R&D (R&D systems, Inc., Minneapolis, MN), mouse and human TNF-α, IL-1β and IL-10 kits from BD Bioscience (BD Biosciences, San Jose, CA) and mouse IFN-β kit from PBL (PBL interferon source, Piscataway, NJ).

RNA Analysis. RNA was purified using the RNeasy kit (Qiagen Sciences, Maryland, USA) and on-column DNA digestion. cDNA was transcribed with the Reverse Transcription System (Promega Corp., Madison, WI). SybrGreen-based real-time quantitative polymerase chain reaction was performed using the iCycler (Bio-Rad Laboratories Inc., Hercules, CA).

Protein quantification. Whole-cell lysates were extracted from liver, as described (20). Equal amounts of proteins were separated on a 10% polyacrylamide gel, and transferred to a nitrocellulose membrane. Interleukin-10 was detected by western blot and immunostaining

with specific primary antibody, followed by horseradish peroxidase (HRP)-labeled secondary antibody (both from Santa Cruz Biotechnology, Santa Cruz, CA). The specific immunoreactive bands of interest were detected by chemiluminescence (Amersham, Piscataway, NJ), and quantified by densitometric analysis.

Histopathological analysis. Sections of formalin-fixed livers were stained with hematoxylin and eosin and analyzed by microscopy.

Isolation of hepatocytes and liver mononuclear cells. Animals received anesthesia with ketamine (100 mg/kg) and xylazine (10 mg/kg); the livers were perfused with saline solution for 5 minutes followed by *in vivo* digestion with Liberase blendzyme 3 (Roche Diagnostics GmbH, Mannheim, Germany) for 5 minutes at 37°C. The hepatocytes were separated by centrifugation at slow speed (350g), liver mononuclear cells (LMNCs) were purified by centrifugation in Percoll gradient.

Isolation of human peripheral blood mononuclear cells. Human peripheral blood mononuclear cells (PBMCs) were separated from blood of healthy volunteers by centrifugation in Ficoll gradient.

In vitro experiments. Primary hepatocytes and LMNCs were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and 1% insulin, transferrin, selenium solution. Primary hepatocytes were seeded in 6-well collagen-coated plates, LMNCs (10^6/insert) were plated in cell-culture inserts with pore diameter 0.4 µm (Becton Dickinson Labware, Bedford, MA). Hepatocytes, LMNCs or co-cultures of hepatocytes with LMNCs were stimulated with LPS (Sigma, St. Louis, MO). IFN-β, IL-10 and TNF-α were measured in supernatants using ELISA. RAW264.7 macrophages were stimulated with LPS, recombinant mouse IFN-α2a (eBioscience, San Diego, CA), recombinant mouse IL-10 (Peprotech Inc., Rocky Hill, NJ) or with anti-mouse IL-10 receptor antibody (Biolegend, San Diego, CA). Human PBMCs were stimulated with LPS, recombinant human IFN-α (PBL, Piscataway, NJ), recombinant IL-10 (Ebioscience, San Diego, CA) or IL-10 receptor antibody (R&D systems, Inc., Minneapolis, MN).

4.2.3. Methods related to the role of probiotics in the pathogenesis of non-alcoholic steatohepatitis

Animal and experimental protocol. C57Bl/6 mice were fed a methionine-choline-deficient (MCD) diet or a methionine-choline-supplemented (MCS) diet (Dyets, Inc. (Bethlehem, PA); the latter control diet was composed of MCD diet supplemented with L-methionine (1.7 g/kg) and choline bitartrate (14.48 g/kg). The mice were fed these diets for a total period of 10 weeks; there were 6-8 mice per experimental group. After a week of adaptation to the new diets, the MCD diet-fed mice were divided into two groups, with one group having its water supply replaced by water containing VSL#3 (VSL Pharmateuticals, Ft. Lauderdale, FL) for the remaining 9 weeks of the experimental feeding. One packet of VSL#3 (450 billion colonies/packet) was mixed in 1L of water and provided to mice instead of drinking water freshly made daily. In pilot experiments we were able to isolate viable VSL#3 bacteria from stool in VSL#3–fed mice; no pathogenic bacteria were isolated from this water. At the end of the 10 week-feeding period some animals were challenged with LPS (0.5mg/kg body weight, i.p) or comparable volumes of saline injected as control.

Histopathological Analysis. Sections of formalin-fixed, paraffin-embedded livers were stained with: 1) hematoxylin and eosin to assess for histologic features of steatohepatitis, and 2) picro-sirius red stain and trichrome stain (Masson’s method) to evaluate for hepatic collagen deposition and fibrosis. The liver sections were also subject to immunohistochemical staining (anti-α–smooth muscle actin antibodies from Abcam, Cambridge, MA).

Biochemical assays. Serum ALT was determined using a kinetic method (D-Tek LLC., Bensalem, PA), serum endotoxin levels were detected using LAL assay (QCL 1000 kit, Cambrex Corporation, Walkersville, MD; detection limit 0.1 EU/ml) and liver triglyceride levels were quantified using a commercial kit (Wako Chemicals Inc., Richmond, VA).
Protein quantification. Cytosolic and nuclear protein fractionation of mouse liver tissue was carried out as previously described (2). Equal amounts of proteins from different stimulation groups were separated on a 10% polyacrylamide gel, and transferred to a nitrocellulose membrane. The proteins of interest were detected by western blot and immunostaining with specific primary antibodies, followed by horseradish peroxidase-labeled secondary antibodies (anti-Collagen and anti-α-SMA from Abcam, Cambridge, MA, secondary-HRP labeled antibodies from Santa Cruz Biotechnology, Santa Cruz, CA). The specific immunoreactive bands of interest were detected by chemiluminescence (Amersham, Piscataway, NJ), and quantified by densitometric analysis.

4.2.4. Methods related to the role of Type I interferons in the pathogenesis of TLR9-associated liver injury

Animals and experimental protocol. The B6.129F2 and C57Bl/6 wild-type (WT) mice were purchased from Jackson Laboratory. IRF7-deficient (IRF7-/-) mice on B6.129F2 background were provided by Tadagatsu Tanaguichi (Tokyo) and type I interferon α/β receptor 1-deficient (IFNAR1-/-) mice on the C57Bl/6 background were the kind gift of Jonathan Sprent (Scripps Research Institute, La Jolla, CA). All animals were 6–8 weeks old. We employed a previously described model of TLR9-associated liver injury induced by administration of TLR9 and TLR2 ligands. After acclimatization, WT, IRF7-/- and IFNAR1-/- mice were injected intraperitoneally (i.p.) with saline or the combination of 2.5 mg/kg unmethylated DNA rich in cytidine-phosphate-guanosine (CpG, ODN1826 murine TLR9 ligand; InvivoGen, San Diego, CA), and 5 mg/kg lipoteichoic acid (LTA, from Staphylococcus aureus; Sigma, Saint Louis, MO). Three days after the above priming stimulus, the mice were injected i.p. with either saline or 0.5 mg/kg lipopolysaccharide (LPS, from Escherichia coli 0111:B4, Sigma, St. Louis, MO) and sacrificed as indicated. Some C57Bl/6 WT mice received a single i.p. injection of 100,000 IU human pegylated interferon alpha-2b (pegIFNα2, Pegintron, Schering, Kenilworth, NJ) two hours prior to LPS. Others were pretreated with recombinant human interleukin-1 receptor antagonist (IL-1ra) 25 mg/kg i.p. every six hours (Anakinra, Amgen, Thousand Oaks, CA) for 24 hours before CpG+LTA, and the treatment with IL-1ra was ongoing until sacrifice. Serum was separated by centrifugation. Livers were snap frozen, stored in RNAlater (Qiagen GmbH, Hilden, Germany) or fixed in 10% neutral-buffered formalin. ALT was quantified by biochemical assay (D-Tek Analytical Laboratories Inc, San Diego, CA).
Histopathology analysis. Sections of formalin-fixed, paraffin-embedded livers were stained with hematoxylin and eosin (H&E), and assessed for inflammatory infiltrate; area of inflammatory infiltrates was calculated with Microsuite (Olympus Soft Imaging Solution GmbH, Munster, Germany) image analysis software in 20 high power fields.
*Isolation of hepatocytes and liver mononuclear cells.*Animals received anesthesia with ketamine and xylazine; the livers were perfused with saline solution followed by in vivo digestion, as we previously described. The hepatocytes and liver mononuclear cells (LMNCs) were purified by centrifugation at slow speed (350g) and in Percoll gradient, respectively.
Phenotype analysis by flow cytometry. Cells were washed in PBS and incubated with anti-CD68 (FITC), anti-CD11c (FITC) or anti-PDCA1 (Alexa Fluor 647) antibodies for 30 minutes on ice. After incubation, cells were washed with PBS, fixed in paraformaldehyde and

analyzed by flow cytometry. All antibodies were from eBioscience (eBioscience, Inc., San Diego, CA).

In vitro cell culture. Primary hepatocytes were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), 1% insulin, transferrin, and selenium supplement on collagen-coated plates (Becton Dickinson Labware, Bedford, MA). Primary LMNCs and murine lymph node endothelial cells SVEC4-10 (ATCC No. CRL-2181) were cultured in DMEM with 10% FBS. Hepatocytes were treated with recombinant murine IFN-α2b (500 IU/mL, eBioscience, San Diego, CA), murine IL-1ra (100 pg/mL, R&D Systems, Minneapolis, MN), murine IL-1β (100 IU/mL, Peprotech Inc., Rocky Hill, NJ) or murine TNF-α (0-100 ng/mL, Peprotech). LMNCs were treated with mouse IFN-α2b or LPS (100 ng/mL, Sigma, St. Louis, MO); SVEC4-10 cells were treated with recombinant murine IFN-α2b.

Hepatocyte cytotoxicity assay. Lactate dehydrogenase (LDH) release from the hepatocyte into the culture supernatants was measured using the LDH-cytotoxicity assay kit (Abcam, Cambridge, MA), and normalized to total LDH (determined after treatment of cells with detergent-based lysis solution).

Cytokine and chemokine measurement. Interleukin 1β and tumor necrosis factor alpha (TNF-α) were measured using ELISA from BD Biosciences (BD Biosciences, San Jose, CA); IL-1 receptor antagonist (IL-1ra) and monocyte chemotactic protein 1 (MCP-1) ELISAs were from R&D (R&D Systems, Minneapolis, MN).

5. Results and discussion

We assessed the role of downstream signals induced by Toll-like receptors in the context of prevalent liver diseases with pronounced activation of innate immunity, such as alcoholic liver disease, non-alcoholic steatohepatitis and TLR9-associated liver injury. We used a broad spectrum of approaches, including population-based genetic testing, *in vivo* animal models and *in vitro* methods to identify pathogenic mechanisms that could be of potential use in diagnosis and therapy of liver diseases.

5.1. The role of allelic variants in the TLR4 -mediated inflammatory pathway in genetic susceptibility to alcoholic liver cirrhosis

We investigated the frequency of functional genetic variants in genes for TLR4, CD14, TNF-α, IL-1β and IL-1ra in 198 Czech patients with ALC and 370 population controls, and in 173 German patients and 331 population controls. In the pilot study involving 100 Czech patients and 180 Czech controls, we observed that patients with ALC showed a significantly higher frequency of the [IL-1RN*2/*2; IL-1B -31T+] diplotype, consisting of alleles that increase production of IL-1b *in vitro* (odds ratio (OR) for ALC = 9.45, 95% CI 1.96 – 45.7). However, we failed to confirm the association in an extended cohort of 198 Czech patients and 370 Czech controls, and in a second cohort of 173 German patients and 331 German controls. We recruited an alternative control group of 109 German heavy drinkers without liver disease, which are expected to have a low frequency of alleles predisposing to ALC and therefore to increase the power of the study to detect a significant association. In spite of including these controls, we did not find an association of the [IL-1RN*2/*2; IL-1B -31T+] diplotype with ALC.

Compared to controls, patients with ALC showed a higher frequency of the *TNFA* -238A allele. However, due to the low population frequency of the *TNFA* -238A allele, our study lacked the power to detect a statistically significant association with ALC. Therefore, we provided our data to a meta-analysis consisting of approximately 500 European patients and 800 European controls (21), which demonstrated a significant association of the *TNFA* -238A allele with ALC. However, the low odds ratio for ALC conferred by this allele (OR 1.47, CI 95% 1.05 – 2.07, p = 0.03) suggested a weak effect in the pathogenesis of ALC.

Analysis of the genetic variants *CD14* -159C/T, *TLR4* c.896A/G and c.1196C/T in our cohort did not show an association with ALC, and failed to replicate positive findings of others (21, 22), which were based on substantially smaller cohorts and therefore subject to increased risk of Type I statistical error (false positivity) (23).

In our study, we did not observe any significant association of the *TNF-A* -238A allele with ALC, contrary to previously published studies that suggested an association of this allele with alcoholic steatohepatitis and cirrhosis. This controversy is likely due to a low frequency of the TNF-A -238A allele (~3%) in the population, low power of individual studies to detect a true association of the risk allele with ALD, and lack of validation cohorts that would confirm the initial positive findings. Our group was asked by dr. M. Marcos, at the University of Salamanca, Spain, to submit the results of the TNF-A -238G/A genotyping to a meta-analysis based on data from a total of 11 studies (Marcos et al., Am J Epidemiol 2009;170(8):948-56, our contribution acknowledged). The authors found a significant association of the TNF-A -238A allele and the risk of alcoholic liver cirrhosis (odds ratio = 1.47, 95% confidence interval: 1.05 - 2.07). In spite of a significant statistical association of

the TNF-A -238G/A with ALD, the low odds ratio (relative risk) suggested its weak biological effect in the pathogenesis of ALC.

Taken together, our results imply that although there is a little doubt that cytokine-mediated immune reactions play a role in the pathogenesis of ALC, hereditary susceptibility caused by variants in key genes involved in TLR4-mediated liver injury seems to be low, or at least such is the case in central European population (24).

5.2. Cell-specific role of the interferon regulatory factor (IRF3) in the pathogenesis of alcoholic liver disease

To study the role of IRF3 in the pathogenesis of alcohol-induced liver injury, we fed wild-type mice (WT), IRF3-deficient mice (IRF3KO), WT mice with transplanted WT bone marrow (WT/WT-BM), WT mice with IRF3KO transplanted bone marrow (WT/IRF3KO-BM) and IRF3KO mice with transplanted WT bone marrow (IRF3KO/WT-BM) with Lieber-DeCarli diet (5% v/v ethanol) or control diet for 4 weeks.

Alcohol feeding resulted in liver injury, steatosis and induction of inflammatory cytokines in WT mice but not in IRF3KO mice. As this finding suggested that IRF3 is involved in the pathogenesis of ALD, we tested the selective contribution of IRF3 in BM-derived cells (WT/IRF3KO-BM mice) and in hepatocytes (IRF3KO/WT-BM mice). We observed that the WT/IRF3KO-BM chimeras showed ameliorated alcohol-induced liver injury and induction of inflammatory cytokines, compared to WT/WT-BM controls, supporting a proinflammatory role of IRF3 in BM-derived cells in ALD (25).

In contrast, IRF3KO/WT-BM chimeras fed with alcohol showed increased liver injury, steatosis and serum inflammatory cytokines, but a significantly decreased expression of Type-I interferons (IFNs) and interleukin-10 (IL-10), an anti-inflammatory cytokine, compared to WT mice. Co-cultures of primary hepatocytes and primary mononuclear cells isolated from WT, IRF3 and Type I IFN receptor α/β–deficient mice demonstrated that hepatocyte-derived IFN-β, dependent of IRF3, induces anti-inflammatory IL-10 and suppressed TNF-α and IL-1β in liver macrophages in paracrine manner.

In conclusion, our results demonstrate a cell-specific role of IRF3 in the pathogenesis of ALD. Activation of IRF3 in bone marrow derived cells increase proinflammatory cytokines and contributes to alcohol-induced liver injury. On the other hand, hepatocyte-specific IRF3 activation and type I IFN induction have protective effects in ALD. Disruption of IRF3 in hepatocytes decreases type I IFN induction and increases liver injury due to dysregulated expression of pro- and anti-inflammatory cytokines (26).

5.3. The role of probiotic diet in the pathogenesis of NASH

We tested the hypothesis that probiotic VSL#3 may ameliorate the methionine-choline-deficient (MCD) diet–induced mouse model of NASH. MCD diet resulted in NASH in WT mice compared to methionine-choline supplemented (MCS) diet feeding evidenced by liver steatosis, increased triglycerides, inflammatory cell accumulation, increased TNF-α levels, and fibrosis. VSL#3 failed to prevent MCD-induced liver steatosis or inflammation. MCD diet, even in the presence of VSL#3, induced up-regulation of serum endotoxin and expression of the TLR-4 signaling components, including CD14 and MD2, MyD88 adaptor, and NFκB activation.

In contrast, VSL#3 treatment ameliorated MCD diet–induced liver fibrosis resulting in diminished accumulation of collagen and α-smooth muscle actin. We identified decreased expression of procollagen and matrix metalloproteinases in mice fed MCD +VSL#3 compared to MCD diet alone. MCD diet triggered up-regulation of transforming growth factor beta (TGF-β), a known profibrotic cytokine. In the presence of VSL#3, the MCD diet–induced expression of TGF-β was maintained; however, the expression of Bambi, a TGF-β pseudoreceptor with negative regulatory function, was increased.

In summary, our data indicate that VSL#3 modulates liver fibrosis but does not protect from inflammation and steatosis in NASH. The mechanisms of VSL#3-mediated protection from MCD diet–induced liver fibrosis likely include modulation of collagen expression and impaired TGF-β signaling (27).

5.4. Protective role of Type I interferons in TLR9-associated liver injury

We investigated the role of Type I interferons (IFNs) and Type I IFN-dependent downstream mediators in regulation of TLR9-associated liver injury. We evaluated liver injury and inflammation in wild-type (WT), IRF7- or IFN α/ß receptor 1 (IFNAR1)-deficient mice in a model of TLR9-dependent injury following intraperitoneal injection of TLR9 (CpG DNA) plus TLR2 (lipoteichoic acid) ligands.

We observed that Type I IFNs were upregulated during TLR9-associated liver injury in WT mice. IRF7- and IFNAR1-deficient mice, that have disrupted Type I IFN induction and signaling, respectively, exhibited exaggerated TLR9-induced liver damage and inflammation, associated with significantly lower recruitment of dendritic cells into the liver, and increased induction of inflammatory cytokines upon *ex vivo* stimulation with LPS. These findings suggested a protective role of Type I IFNs in the liver by limiting liver inflammation and injury and implied involvement of Type I IFN-dependent anti-inflammatory factors.

Interleukin 1-receptor antagonist (IL-1ra) is a Type I IFN-regulated antagonist of the pro-inflammatory cytokine IL-1β. TLR9 drives both IL-1β and IL-1ra. We identified decreased IL-1ra in IRF7- and IFNAR1-deficient mice downstream of TLR9, compared to WT mice, suggesting a dysbalance in IL-1β/IL-1ra signaling and preferential proinflammatory activation. In vivo administration of Type I IFN to WT mice induced IL-1ra and significantly ameliorated TLR9-associated liver injury. The protective role of IL-1ra was confirmed in vivo where administration of recombinant IL-1ra protected against TLR9-associated liver injury suggesting that the anti-inflammatory effect of Type-I IFNs could be mediated by IFN-dependent induction of IL-1ra.

Our findings imply that the endogenous anti-inflammatory signaling induced by Type I IFNs and mediated by IL-1ra regulates the extent of TLR9-induced liver damage, and support

the indispensable role of Type I interferon signaling in immune-mediated liver injury. Our data also suggest a potential role for IL-1ra in therapy of TLR9-associated liver diseases (28).

6. Conclusions

Within the subject of Toll-like receptors in the pathogenesis of liver injury, our work resulted in and contributed to the following points:

A. We determined that hereditary susceptibility caused by common variants in genes involved in TLR4-induced pathogenesis of alcoholic liver cirrhosis is low in central European population.

a. Small or pilot allelic association studies frequently result in positive findings that are not replicable in larger cohorts.
b. Performing allelic association studies in meta-analyses or multicentric studies increases the probability of detecting a true positive association; however, statistically significant results from such studies may be of low biological significance in the context of stronger demographic and clinical determinants.

B. We showed that hepatocyte-derived Type I interferons play anti-inflammatory role in the pathogenesis of alcohol-induced liver disease in mice.

a. The pathogenesis of alcohol-induced liver disease is critically dependent on the interferon regulatory factor 3 (IRF3), an alternative downstream mediator of TLR4 activation.
b. IRF3 in bone-marrow derived cells in the liver has pro-inflammatory role in alcohol-induced liver disease.
c. Hepatocyte-specific IRF3 is protective in alcohol-induced liver disease by means of induction of Type I IFNs that induce the anti-inflammatory IL-10 in mononuclear cells, thus diminishing the extent of alcohol-induced liver inflammation and injury.
d. In the context of alcohol-induced liver disease, the crosstalk between hepatocytes and bone-marrow derived cells is critical in regulating the extent of liver inflammation and damage induced by ethanol.

C. We demonstrated a differential effect of probiotic diet on inflammation and liver fibrosis in non-alcoholic steatohepatitis (NASH) model in mice.

a. VSL#3 probiotic diet did not show any effect on TLR4-dependent inflammatory signaling, inflammation and steatosis in NASH.
b. VSL#3 probiotic diet significantly ameliorated NASH-associated liver fibrosis. This effect likely involved modulation of collagen expression and impaired TGF-β signaling.
c. Our results suggest that, at least in the NASH model, the benefit of the VSL#3 treatment on fibrosis may occur even in the absence of significant changes in markers of inflammation and fat in the liver.

D. We identified a protective role of Type I interferons in immune-mediated liver injury induced by TLR9 signaling.

a. Type I interferons are significantly induced in TLR9-associated liver diseases
b. Absence of Type I interferon induction or signaling substantially aggravated TLR9-associated liver inflammation and injury, increased production of inflammatory

cytokines by liver mononuclear cells, and decreased the expression of interleukin 1-receptor antagonist, which is a Type I interferon-dependent anti-inflammatory cytokine.

c. Our results demonstrated a critical anti-inflammatory and protective role of Type I interferons and Interleukin-1 receptor antagonist in immune-mediated liver injury and suggest potential therapeutic implications.

7. References

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27. Velayudham A, Dolganiuc A, Ellis M, Petrasek J, Kodys K, Mandrekar P, et al. VSL#3 probiotic treatment attenuates fibrosis without changes in steatohepatitis in a diet-induced nonalcoholic steatohepatitis model in mice. Hepatology 2009;49:989-997.
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8. List of author's publications, awards and presentations

a. List of author's publications related to the topic of the Ph.D. thesis

i. Original articles

1. **Petrasek J**, Hubacek JA, Stickel F, Sperl J, Berg T, Ruf E, et al. Do common genetic variants in endotoxin signaling pathway contribute to predisposition to alcoholic liver cirrhosis? *Clin Chem Lab Med* 2009;47:398-404. **IF: 1.89**
2. **Petrasek J**, Dolganiuc A, Nath B, Hritz I, Kodys K, Catalano D, et al. Hepatocyte-specific IRF3 and Type I interferons are protective in alcohol-induced liver injury in mice via cross-talk with macrophages. 2010; Manuscript submitted.
3. Velayudham A, Dolganiuc A, Ellis M, **Petrasek J**, Kodys K, Mandrekar P, et al. VSL#3 probiotic treatment attenuates fibrosis without changes in steatohepatitis in a diet-induced nonalcoholic steatohepatitis model in mice. *Hepatology* 2009;49:989-997. **IF: 11.56**
4. **Petrasek J**, Dolganiuc A, Csak T, Kurt-Jones E, Szabo G. Type I Interferons protect from Toll-like receptor 9-associated liver injury and regulate IL-1 receptor antagonist in mice. *Gastroenterology* 2010; *doi. 10.1053/j.gastro.2010.08.020*. **IF: 12.89**

ii. Review articles

5. **Petrasek J**, Mandrekar P, Szabo G. Toll-like receptors in the pathogenesis of alcoholic liver disease. Gastroenterology research and practice 2010. doi:10.1155/2010/710381.
6. **Petrasek J.** Genetické faktory v patogenezi alkoholické nemoci jater. In: Špičák, J et al. Novinky v gastroenterologii a hepatologii. Grada publishing 2006, pp. 87-118. ISBN: 978-80-247-1783-8

b. List of author's publications not related to the topic of the Ph.D. thesis

1. Dolganiuc A, **Petrasek J**, Kodys K, Catalano D, Mandrekar P, Velayudham A, et al. MicroRNA expression profile in Lieber-DeCarli diet-induced alcoholic and methionine choline deficient diet-induced nonalcoholic steatohepatitis models in mice. Alcohol Clin Exp Res 2009;33:1704-1710. **IF: 3.33**
2. **Petrasek J**, Jirsa M, Sperl J, Kozak L, Taimr P, Spicak J, et al. Revised King's College score for liver transplantation in adult patients with Wilson's disease. Liver Transpl 2007;13:55-61. **IF: 4.22**
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17. **Petrasek J** et al. Molekulární diagnostika nejčastějších dědičných chorob trávicí soustavy: II. Kolorektální karciniom a choroby pankreatu. Praktický lékař, 2006, vol. 86, no. 11, p. 654-656.
18. **Petrasek J** et al. Polymorfismy v genovém clusteru pro interleukin 1: rizikový faktor alkoholické jaterní cirhózy. Čes. a Slov. Gastroent. a Hepatol. 2007, vol. 61, no. 2, p. 26 – 31.

c. List of author's awards

2010 The Research Society on Alcoholism award. 33rd Annual RSA Scientific Meeting, San Atonio, Texas, U.S.A. Oral presentation.

2010 Cena Jana Opletala (Jan Opletal Award), 1st Faculty of Medicine, Charles University Prague, Czech republic. Distinguished Ph.D. student award.

2010 American Gastroenterological Association award (oral presentation). Digestive Disease Week (DDW).

2010 European Association For The Study Of The Liver young investigator travel award. Monothematic conference: Signaling in the Liver

2009 American Gastroenterological Association award (oral presentation). Digestive Disease Week (DDW).
2009 American Association for the Study of Liver Diseases award (poster of distinction). AASLD Liver meeting.
2008 European Association For The Study Of The Liver young investigator travel award. EASL liver week.
2008 American Gastroenterological Association award (poster of distinction). Digestive Disease Week (DDW).
2008 Czech hepatology society award (best original article published in an international journal in 2007)
2008 1. cena Nadačního fondu Scientia (Scientia foundation Distinguished Ph.D. student award). 1st Faculty of Medicine, Charles University Prague.
2007 Slovak gastroenterological society for the best poster presentation. Gastroforum meeting 2007
2007 European Association For The Study Of The Liver young investigator travel award. EASL liver week.
2007 American Gastroenterological Association award (poster of distinction). Digestive Disease Week (DDW).
2006 European Association For The Study Of The Liver young investigator travel award. EASL liver week.
2006 Falk foundation award for the 2nd best poster presentation. Falk Symposiun No. 156: genetics in Liver Diseases.
2006 8th European Bridging Meeting in Gastroenterology Award for the best oral presentation.

d. List of author's presentations

1. **Petrasek J**, Csak T, Kurt-Jones, E, Szabo, G. Interferon regulatory factor 3 signaling promotes liver fibrosis in mice and regulates the chemokine ligand 21 via Type-I interferons. The Liver Meeting 2009, American Association for the Study of Liver Disease (AASLD), November 2010, Boston, Massachusetts, U.S.A. **Poster presentation.**
2. **Petrasek J**, Dolganiuc A, Nath B, Hritz I, Kodys K, Catalano D, Mandrekar P, Szabo G. Hepatocyte-specific IRF3 signaling protects from alcohol-induced liver injury in mice through regulation of monocyte/macrophage-derived cytokines via Type-I interferons. The 33rd Annual RSA Scientific Meeting, 28.6.2010, San Atonio, Texas, U.S.A. **Oral presentation**.
3. **Petrasek J**, Dolganiuc A, Kurt-Jones, E, Szabo, G. Type I Interferons Regulate IL-1 Receptor Antagonist and Protect from Toll-like Receptor 9-Mediated Liver Injury in Mice. *Digestive Disease Week 2010 (DDW)*, 2.5.2010, New Orleans, U.S.A. **Oral presentation.**
4. **Petrasek J**, Dolganiuc A, Nath B, Hritz I, Kodys K, Catalano D, Mandrekar P, Szabo G. Protective effect of hepatocyte-specific Type-I interferon in alcohol-mediated liver injury. The Henry and Lillian Stratton basic research conference of the American Association for the Study of Liver Disease (AASLD), 12.9.2009 – 14.9.2009, Atlanta, Georgia, U.S.A. **Poster.**
5. **Petrasek J**, Dolganiuc A, Nath B, Hritz I, Kodys K, Catalano D, Mandrekar P, Szabo G. Hepatocyte-specific IRF3 signaling protects from alcohol-induced liver injury in mice through regulation of monocyte/macrophage-derived cytokines via Type-I interferons.

The Liver Meeting 2009, American Association for the Study of Liver Disease (AASLD), 30.10.2009 - 3.11.2009, Boston, Massachusetts, U.S.A. **Poster of distinction.**

6. **Petrasek J**, Dolganiuc A, Kurt-Jones, E, Szabo, G. Type I Interferon Induction and Signaling Protect from Liver Injury and Granulomas Through Regulation of IL-1 Receptor Antagonist and Dendritic Cell Recruitment in Mice. Digestive Disease Week 2009 (DDW), 2.6.2009, Chicago, U.S.A. **Oral presentation.**
7. Csak T, Dolganiuc A, Nath B, **Petrasek J**, Mandrekar P, Szabo G. Non alcoholic steatohepatitis sensitizes to poly I:C induced liver damage and leads to decreased antiviral immune response by reducing mitochondrial association of the antiviral signaling protein (MAVS). The Liver Meeting 2009, American Association for the Study of Liver Disease (AASLD), 30.10.2009 - 3.11.2009, Boston, Massachusetts, U.S.A. **Poster of distinction.**
8. Nath B, Levin I, Csak T, **Petrasek J**, et al. Hypoxia inducible factor-1α is a determinant of lipid accumulation and liver injury in alcoholic steatosis. The Liver Meeting 2009, American Association for the Study of Liver Disease (AASLD), 30.10.2009 - 3.11.2009, Boston, Massachusetts, U.S.A. **Oral presentation.**
9. Marcos M, Bala S, Kodys K, Catalano D, **Petrasek J, et al** . MicroRNA-155 in Kupffer cells mediates increased TNF-alpha production in alcoholic liver disease. *The Liver Meeting 2009, American Association for the Study of Liver Disease (AASLD),* 30.10.2009 - 3.11.2009, Boston, Massachusetts, U.S.A. **Poster.**
10. **Petrasek J**, et al. Temporary nasobiliary drainage induces relief from severe long-lasting pruritus in drug-induced canalicular cholestasis. *43rd Annual Meeting of the European Association for The Study Of The Liver (EASL)*, Milano, Italy, 23. - 27. 4. 2008. **Poster.**
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